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PRC17, a Novel Oncogene Encoding a Rab GTPase-activating Protein, Is Amplified in Prostate Cancer

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Abstract

We used cDNA-based genomic microarrays to examine DNA copy number changes in a panel of prostate tumors and found a previously undescribed amplicon on chromosome 17 containing a novel overexpressed gene that we termed *prostate cancer gene 17* (*PRC17*). When overexpressed in 3T3 mouse fibroblast cells, *PRC17* induced growth in low serum, loss of contact inhibition, and tumor formation in nude mice. The *PRC17* gene product contains a GTPase-activating protein (GAP) catalytic core motif found in various *Rab/Ypt* GAPs, including RN-Tre. Similar to RN-Tre, we found that PRC17 protein interacts directly with Rab5 and stimulates its GTP hydrolysis. Point mutations that alter conserved amino acid residues within the PRC17 GAP domain abolished its transforming abilities, suggesting that GAP activity is essential for its oncogenic function. Whereas *PRC17* is amplified in 15% of prostate cancers, it is highly overexpressed in approximately one-half of metastatic prostate tumors. The potent oncogenic activity of *PRC17* is likely to influence the tumorigenic phenotype of these prostate cancers.

Introduction

Despite the widespread attention given to the study of prostate cancer, only modest progress has been made in obtaining a comprehensive and consistent picture of the genetic changes that underlie prostate cancer development. The most commonly involved tumor suppressor genes appear to be *PTEN* and *p53*, although there are inconsistent findings regarding the frequency of inactivation of these two genes in prostate cancer (1, 2). No human oncogenes that are activated by point mutations or genomic amplification in other solid tumor types, including *RAS*, *MYC*, and *HER2*, have been found to be frequently mutated or amplified in prostate cancer (3–5).

Previous analysis of prostate cancers with comparative genomic hybridization suggested that there are undiscovered amplified oncogenes located within chromosomal regions that undergo a significant DNA copy number increase (6, 7). The chromosomal regions identified by comparative genomic hybridization are too large to allow efficient identification of underlying amplified oncogenes, and therefore much effort has been made to develop microarray-based comparative genomic analysis methods that can define smaller regions of amplification (8, 9). One such method utilizes tumor and normal genomic DNA hybridized to cDNA microarrays to detect amplified regions in human tumor samples (10). In this report, we used microarrays containing 31,104 cDNAs to perform a genome-wide survey for amplification in 22 primary prostate tumor samples. We detected a novel amplicon on 17q12 in 1 of the 22 samples. A novel oncogene candidate, *PRC17*, was identified from this amplicon, and we report here on the initial characterization of its oncogenic and biochemical functions.

Materials and Methods

Prostate Tumor Samples. Twenty-two primary prostate tumor samples were purchased from BioClinical Partners (Boston, MA). Fifteen metastatic tumors were obtained from "warm autopsies" (11). Genomic DNA was extracted from the tumor samples using the proteinase K method, and total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA).

Microarrays, Hybridization, and Analysis. Sequence-verified human cDNA clones (a total of 31,104) were obtained from Research Genetics (Huntsville, AL). The cDNAs were amplified using the PCR. The PCR products were purified with Millipore filters (Bedford, MA), resuspended in 3× SSC, and printed with an Intelligent Automation Systems robotic arrayer (Cambridge, MA) onto Corning Gamma Amino Propyl Silane (GAPS) slides (Corning, NY). Labeling and hybridization were performed essentially as described previously (10). Microarrays were scanned using an Axon GenePix 4000 scanner and analyzed with GenePix Pro 2.0 software (Union City, CA). The fluorescence ratios representing the amount of tumor DNA relative to normal DNA were converted into chromosomal plots essentially as described previously (10). The chromosomal position of approximately 95% of the cDNAs was determined by BLAT analysis of genomic sequence draft.[2]

Fluorescence-based Real-Time PCR. Fluorogenic Taqman probes were designed based on expressed sequence tags or BAC[3] sequences. Changes in the DNA copy number in the tumor cells were quantified using the ABI PRISM 7700 Sequence Detection System from Applied Biosystems (Foster City, CA) through 40 cycles. Absolute mRNA levels for the genes of interest were also determined by real-time reverse transcriptation-Taqman. β-Actin probe was used as reference.

cDNA Cloning, Mutagenesis, and Generation of Cell Lines Overexpressing Wild-type and Mutant PRC17. Forward primer 5′-cgggatccaccaccatggacgtggtagaggtcg-3′ and reverse primer 5′-ccgctcgagctagaagcctggaggaac-3′ were used to PCR amplification of full-length PRC17 cDNA from Clontech fetal brain and fetal lung cDNA library (Palo Alto, CA) using *Pfu* Taq polymerase. The PCR product was gel-purified and cloned into *Bam*HI- and *Xho*I-digested retroviral vector, pLPC. Retroviral-mediated gene transfer into NIH3T3 cells was performed as described previously (12).

Site-directed mutagenesis was performed to change Arg^{107} and Asp^{148} to Ala using the QuikChange Site-directed mutagenesis kit according to the manufacturer's protocol (Stratagene, La Jolla, CA). The coding region of either wild-type or mutant PCR17 was cloned into retroviral vector pLPC.

Cell Proliferation, Focus Formation, and Tumorigenicity Assays. Cell proliferation was assayed using the Celltiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) according to the manufacturer's instructions. The absorbance at 490 nm was recorded using an ELISA reader.

For the focus formation assay, NIH3T3 cells were transfected with pLPC vector or pLPC-PRC17. Forty-eight h after transfection, cells were split 1:3 and grown in medium containing 5% serum until confluent. The formation of foci was inspected visually after 2 weeks of culture in low serum.

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[2] http://genome.ucsc.edu.

[3] The abbreviations used are: BAC, bacterial artificial chromosome; GAP, GTPase-activating protein; RT-PCR, reverse transcription-PCR.

Stable 3T3 cells expressing either wild-type or mutant PRC17 were harvested and resuspended in MEM. The cell suspension (5×10^6 cells/injection) was injected s.c. into athymic nude mice. Mice were observed weekly for the visual appearance of tumors at injection sites, and tumor sizes were measured each week.

Expression and Purification of Recombinant Proteins. To generate NH_2-terminal His-PRC17 proteins, cDNAs encoding *PRC17* and its mutant were subcloned into either pET28a prokaryotic or pFASTBAC HTb baculoviral expression vector. The resulting fusion proteins were expressed in either B21 *Escherichia coli* or sf9 insect cells and purified on a Ni^{2+}-NTA agarose column. The cDNAs for human *Rab4, Rab5,* and *Rab11* were cloned by RT-PCR using human brain RNA. The coding regions were then subcloned into pET28a vector and expressed and purified as described for PRC17 protein.

GAP Assay. Recombinant Rab proteins (30 ng) were preloaded with [γ-^{32}P]GTP (10 μCi, 6000 Ci/mmol; New England Nuclear) in 20 μl of 20 mM Tris-HCl (pH 7.6), 0.1 mM DTT, 25 mM NaCl, and 4 mM EDTA for 10 min at 30°C. $MgCl_2$ was then added to a final concentration of 17 mM. Three μl of the preloaded protein were diluted with 20 mM Tris-HCl (pH 7.6), 0.1 mM DTT, and 100 ng of PRC17 protein in a final volume of 30 μl. NaCl was added to a final concentration of 150 mM, and the mixture was incubated at 22°C. Five-μl samples were removed after 5 min, diluted to 1 ml with cold buffer A (50 mM Tris-HCl, 5 mM $MgCl_2$, and 50 mM NaCl), and filtered through nitrocellulose filters (pre-wet with buffer A). Filters were washed with 10 ml of cold buffer A, dried, and counted.

Immunoprecipitation and Western Analysis. Cell lysate was prepared from NIH3T3 cells stably expressing either wild-type or mutant PRC17. Cells were lysed in radioimmunoprecipitation assay buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS), and immunoprecipitated with anti-Rab5 antibody (Santa Cruz Biotechnology) in the presence of protein A/G-agarose. After extensive washing with radioimmunoprecipitation assay buffer, the immunocomplex was resolved by SDS-PAGE and blotted. The membrane was incubated with anti-PRC17 antibody and detected by enhanced chemiluminescence. A rabbit polyclonal antibody directed against a unique COOH-terminal peptide in PRC17 (NH_2-PSTDSDQGTPFRARDEQPC-OH) was generated by Antibody Solution (Palo Alto, CA).

Results

Identification of a Novel 17q12 Amplicon. We analyzed a total of 22 primary prostate cancer samples for DNA amplifications by hybridizing their genomic DNA, labeled with one fluorescent dye, along with an equal amount of normal DNA, labeled with a distinct fluorescent dye, to cDNA microarrays. Twenty-one of these 22 tumor samples showed no significant amplifications by this method. One sample displayed a single amplicon that contained four closely linked cDNAs (R47893, AA780746, H62985, and AA677522), all of which displayed higher copy number (approximately 2-fold) in the prostate cancer sample than in normal DNA. These four cDNAs were located within a 150-kbp region within 17q12, based on the April 2001 assembly of the Human Genome Project working draft sequence.[2] *HER2*, which is approximately 4 million bp telomeric to these sequences, was not highly amplified in this prostate tumor. We then determined the frequency of amplification of this region in primary prostate and breast cancers using more sensitive PCR-based Taqman analysis. Three of 20 primary prostate cancers displayed >2.5-fold amplification, and the highest level of amplification was 15-fold in the original tumor. In addition, 4 of 26 breast cancer cell lines showed significant amplification ranging from 4-fold to >20-fold.

To determine the candidate target genes that provide cancer cells with the selective growth advantage underlying this genomic amplification, we examined a panel of the most highly amplified tumors and cancer cell lines to determine the epicenter of maximal amplification. We measured DNA copy number with 13 quantitative PCR probes designed from sequences distributed throughout a 0.6-million bp region surrounding the four cDNAs that were amplified in the original tumor (Fig. 1*A*). The epicenter is defined most clearly by the breast cancer cell line MDAMB330 and the primary prostate tumor pp480, with the highest level of amplification contained within an approximately 200-kb region (Fig. 1*A*). There are only two previously known cytokines genes located in this region: *SCYA4* and *SCYA3L1*. Neither was expressed in the 12 breast and 4 prostate cancer cell lines we tested.

Identification and Characterization of *PRC17*. To identify other genes within the epicenter, we examined the nucleotide sequences of the underlying BAC sequences using the Basic Local Alignment Search Tool (BLASTX).[4] We observed 13 regions containing homology to the *Tre2* oncogene (E value < 0.01). Using sequence comparison, available expressed sequence tags, and the exon prediction program Genscan (13), we were able to assemble a putative novel gene comprised of 13 exons. We named it *PRC17*. We were able to clone the full-length *PRC17* cDNA, which predicts a protein of 549 amino acids and shares 84% amino acid identity with *Tre-2*. However, the ubiquitin hydrolase domain encoded by a region near the end of the *Tre-2* open reading frame is not present in *PRC17*. The predicted PRC17 protein contains a conserved GAP domain (also called TBC or TrH) found in the yeast cell cycle regulators Bub2 and cdc16 as well as in Rab/Ypt-specific GTPase activators Gyp7, Gyp9, and RN-Tre (14–16). The sequence alignment of the Rab/Ypt GAP domain of PRC17, Tre-2, and RN-Tre is shown in Fig. 1*B*.

We examined *PRC17* mRNA expression in several normal human tissues by both Northern analysis and quantitative RT-PCR. Message was detected at low levels in all tissues examined. The expression pattern of *PRC17* correlated with the amplification status in breast cancer cell lines and primary breast tumors. In one primary breast tumor that showed 5-fold genomic amplification, quantitative RT-PCR analysis revealed 5-fold overexpression. Four breast cancer cell lines, MDAMB330, ZR75-30, BT474, and AlaB, showed amplification at 18-, 6-, 7-, and 3.5-fold, respectively. They also displayed overexpression of 66-, 18-, 6-, and 2.5-fold, respectively. The genomic amplification of the *PRC17* gene in MDAMB330 and BT474 was also confirmed by Southern analysis (Fig. 1*C*). Overexpression of *PRC17* at the mRNA level in these two cell lines was also validated by Northern analysis (data not shown). However, due to the poor quality of RNA in our primary prostate tumors, the expression of *PRC17* in the amplified samples was impossible to quantitate.

High-level overexpression ranging from 9- to 200-fold (average, 45-fold) was detected in 8 of the 15 metastatic prostate tumors tested in the absence of gene amplification. An anti-PRC17 rabbit polyclonal antibody was generated against a COOH-terminal peptide that was unique to PRC17. The levels of endogenous PRC17 protein in metastatic prostate samples were determined by Western blotting (Fig. 1*D*). The two metastatic prostate tumors with high levels of *PRC17* gene expression also showed overexpression of PRC17 protein. The endogenous protein and recombinant protein migrated at the same level (see Fig. 2*A*).

PRC17 Interacts with Rab5 and Activates Rab5 GTPase Activity. The GAP domain of *PRC17* is found in mammalian RN-Tre, a Rab5 GAP (14). To test whether PRC17 acts as a Rab5 GAP, we initially determined whether PRC17 and Rab5 interact with each other *in vivo*. We established cell lines that stably express either wild-type PRC17 or a mutant form of PRC17 in which two conserved amino acids were substituted by alanines. Point mutations of the corresponding amino acids on RN-Tre have been shown to abolish GAP activity of that protein (17). The expression of both wild-type and mutant

[4] http://www.ncbi.nlm.nih.gov/BLAST.

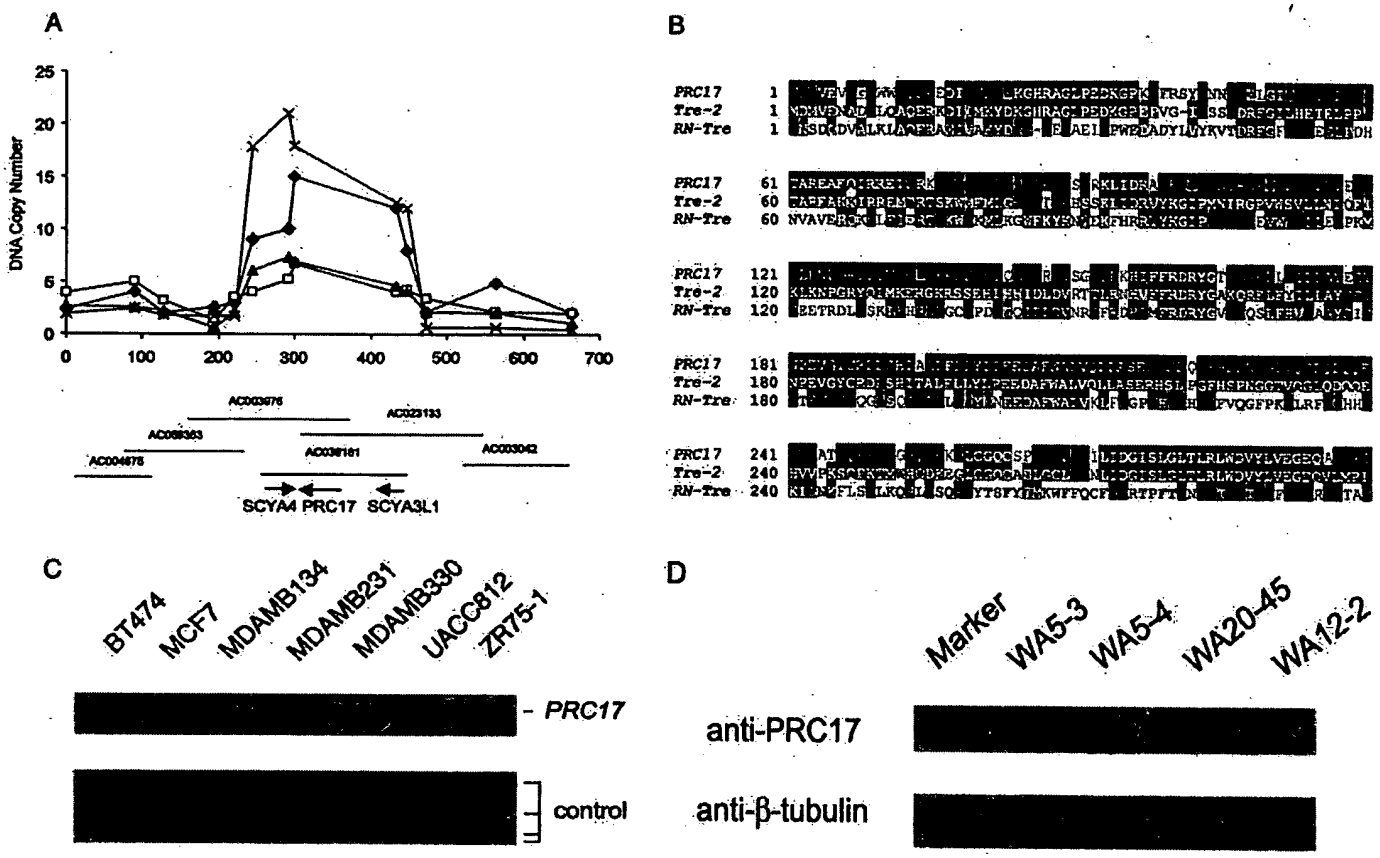

Fig. 1. Identification of PRC17. *A*, epicenter mapping of the 17q12 amplicon. The DNA copy numbers of breast cancer cell lines MDAMB330 (x) and BT474 (▲) and two primary prostate samples, pp480 (♦) and pp486 (□), were evaluated using quantitative PCR. The fold of amplification was plotted against the physical positions of fluorogenic Taqman probes. The regional BAC contig was assembled based on the sequence homology and physical presence of BAC ends and agrees with the December 2001 University of California Santa Cruz assembly (http://genome.ucsc.edu). *B*, sequence alignments of the TrH domain among *PRC17* (GenBank accession number AF540953), *Tre-2* (GenBank accession number X63546), and *RN-Tre* (GenBank accession number NM_014688). The identical residues are highlighted in *black*, and the conserved residues are highlighted in *gray*. *C*, Southern blot analysis on breast cancer cell lines BT474, MCF7, MDAMB134, MDAMB231, MDAMB330, UACC812, and ZR75-1 after genomic DNA from these cell lines was digested with *Hin*dIII. Full-length PRC17 cDNA was used as probe. The same blot was probed with a control DNA from chromosome 7. *D*, detection of endogenous PRC17 protein in metastatic prostate tumor samples. PRC17 protein levels in metastatic prostate tumor samples (WA5-3, WA5-4, WA20-45, and WA12-2) were measured by Western blot. β-Tubulin antibody was used as a control for loading efficiency.

PRC17 in established cell lines was verified by Western blot analyses (Fig. 2*A*).

Using these cell lines, we performed immunoprecipitation with anti-Rab5 antibody. As shown in Fig. 2*B*, *top panel*, PRC17 protein was detected in immunocomplexes from cells expressing either wild-type or mutant *PRC17*, whereas no PRC17 protein was present in the immunocomplex from cells expressing the vector. Western blot analysis showed that Rab5 was present in all cell lines (Fig. 2*B*, *bottom panel*). These results suggest that PRC17 interacts with Rab5 *in vivo* and that point mutations within the PRC17 Rab GAP domain have no effect on protein-protein interaction between PRC17 and Rab5.

To test whether PRC17 is a Rab5 GAP, recombinant PRC17 and various Rabs were expressed in and purified from sf9 insect cells or *E. coli* (Fig. 2*C*). We then performed GTP hydrolysis assays using recombinant PRC17 and Rab5 proteins (Fig. 2*D*). As shown in Fig. 2*D*, about 70% of Rab5 protein was GTP-bound after a 5-min incubation in the absence of PRC17 protein. The addition of wild-type PRC17 resulted in a 30% reduction of GTP-bound Rab5, whereas the addition of the mutant form of PRC17 had no effect on Rab5 GTP hydrolysis. These results demonstrate that PRC17 activates Rab5 GTPase activity and that point mutations within the GAP domain abolished GAP activity of PRC17. To test whether this GAP activity is specific to Rab5, two additional Rabs, *Rab4* and *Rab11*, were also cloned, expressed, and purified from *E. coli* (Fig. 2*C*). As shown in Fig. 2*E*, PRC17 has no effect on the intrinsic GTPase activity of either Rab4 or Rab11.

PRC17 Confers Growth Advantage in Low Serum. To characterize the oncogenic properties of PRC17, we first tested the role of PRC17 in the ability to induce proliferation in low-serum concentrations using a nonradioactive cell proliferation assay. As shown in Fig. 3*A*, when cells were cultured in 0.5% serum, 3T3 cells expressing either vector alone or the mutant form of PRC17 stopped dividing after 48 h, whereas cells expressing wild-type PRC17 proliferated rapidly in the low serum, resulting in a 3-fold increase in cell number after 72 h in culture. These results suggest that overexpression of PRC17 confers a growth advantage in low serum and that this function requires a functional GAP domain.

PRC17 Induces Foci Formation and Increases Saturation Density. 3T3 cells were transfected with either vector alone or PRC17 and then grown for 2–3 weeks in a standard focus formation assay. Only the PRC17-transfected cells developed foci after 2 weeks in culture (Fig. 3*B*). This result suggests that cells overexpressing PRC17 acquired the property of reduced contact inhibition. This observation was further confirmed by measuring the saturation density. The stably transfected 3T3 cells were monitored for growth by counting cell numbers for a period of 2 weeks. With the control transfectants, when the cells reached confluence (at day 4), the total cell number started to decrease. However, with the PRC17 transfectants, the cells kept dividing at a slow rate even after they had reached confluence. At the end of the 2-week period, the number of PRC17 cells was nearly three times greater than that of control cells (data not shown).

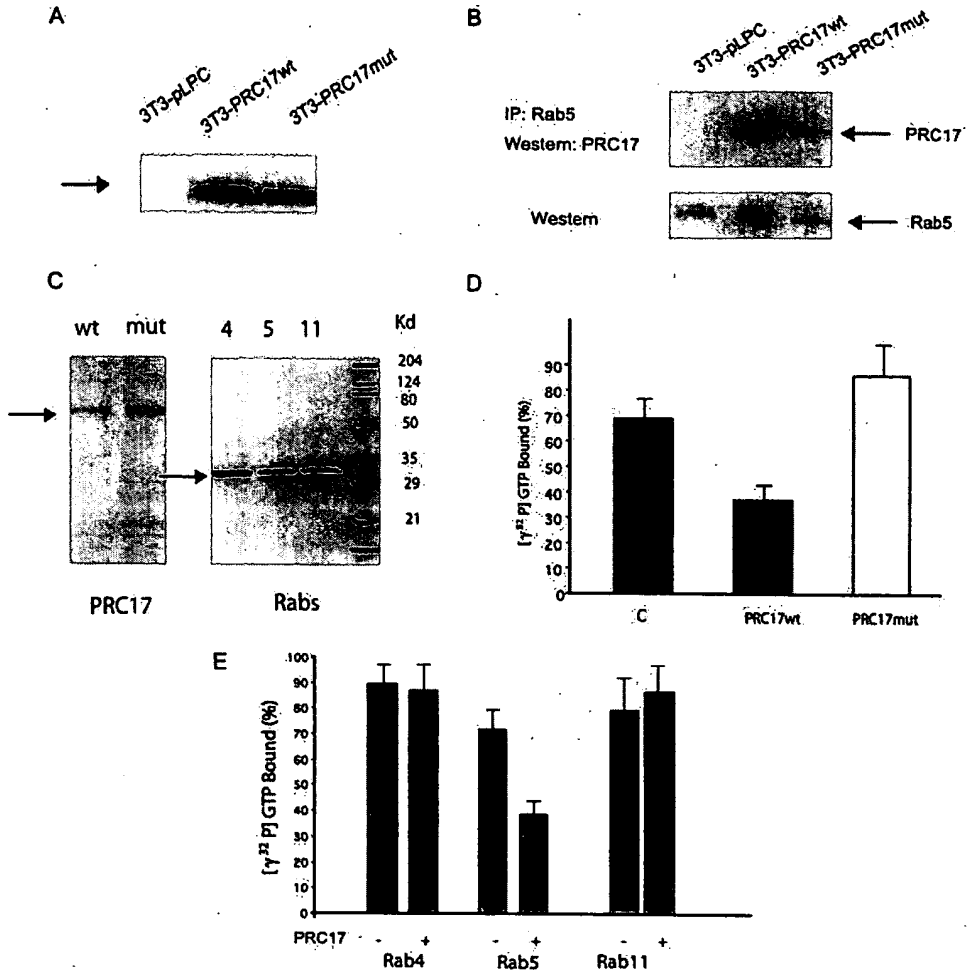

Fig. 2. PRC17 interacts with Rab5 and stimulates GTP hydrolysis on Rab5. *A*, expression of wild-type and mutant PRC17 protein in 3T3 cells. Western blot analysis was performed using whole cell lysate from the indicated cell lines and anti-PRC17 antibody. *B*, interaction between PRC17 and Rab5. Whole cell lysates from indicated cell lines were immunoprecipitated with anti-Rab5 antibody, and the immunocomplexes were analyzed by Western blot using anti-PRC17 and anti-Rab5 antibody. *C*, purification of PRC17 (from sf9 insect cells) and Rab proteins (from *E. coli*) and Coomassie Blue staining of purified recombinant proteins. *D*, GTP hydrolysis assay. Rab5 was loaded with [γ-^{32}P]GTP and incubated with BSA (*C*) or purified wild-type or mutant PRC17. GAP activity was expressed as the percentage of nonhydrolyzed [γ-^{32}P]GTP that remained bound to the filters, relative to the radioactivity at time 0. Values are the mean of three independent experiments. *E*, GTP hydrolysis assay of PRC17 on Rab5, Rab4, and Rab11. Assay conditions were the same as those described above. Values are the mean of three independent experiments.

Tumorigenesis of PRC17 in Nude Mice. To determine whether PRC17 is tumorigenic *in vivo*, 3T3 cells expressing vector alone or wild-type or mutant PRC17 were injected s.c. into athymic nude mice. The protein levels for the wild-type and mutant PRC17 were comparable in the stable lines (Fig. 2*A*). As shown in Fig. 3*C*, all animals injected with 3T3 cells expressing wild-type PRC17 developed large tumors within 4 weeks. No mouse injected with cells expressing vector alone developed tumors. Of the mice injected with cells expressing mutant PRC17, only one developed a small tumor after 4 weeks. These results suggest that PRC17 is a potent transforming gene *in vivo* and that GAP activity is required for its transforming function.

Discussion

In this study, we have used cDNA microarray technology to identify a novel amplified oncogene, *PRC17*. *PRC17* is the only overexpressed gene in the epicenter of the amplicon. The *PRC17* gene is highly homologous to the *Tre-2* oncogene (85% amino acid identity), which also confers tumorigenicity to NIH3T3 cells (18). However, it was assumed that the oncogenic activity of *Tre-2* resulted from the portion of its coding sequence that potentially encodes a deubiquitinating enzyme (19). That region of *Tre-2* is not found in *PRC17*, and therefore it appears more likely that the oncogenic function of *Tre-2* results from the region it shares with *PRC17*.

Both the *PRC17* and *Tre-2* gene products share 37% amino acid identity to the Rab/Ypt GAP domain found in *RN-Tre* (14). RN-Tre is a Rab5 GAP that plays an important role in the regulation of epidermal growth factor receptor internalization (17). We demonstrated that PRC17 also acts as a Rab5 GAP. We also showed that mutating the conserved amino acids Arg^{107} and Asp^{148} of the Rab/Ypt GAP domain of PRC17 abolished the ability of PRC17 to activate GTP hydrolysis on Rab5, although point mutations of these residues did not affect interaction with Rab5. These results agree with the previous study with RN-Tre, in which point mutations on the corresponding amino acid residues led to loss of GAP activity (17). Importantly, these point mutations of the conserved amino acids within the *PRC17* GAP domain resulted in loss of its transforming activity. This suggests that PRC17 oncogenic function is closely related to its role in modulating Rab5, and we propose that the highly homologous *Tre-2* oncogene induces tumorigenicity in a similar fashion.

Rab5 is involved in regulating the formation of endosomes, one of the earliest stages of receptor-mediated endocytosis (20). Dominant negative Rab5, presumably by promoting the formation of inactive, GDP-bound endogenous Rab5, inhibits receptor endocytosis (21). Constitutively activated Rab5, which is relatively more GTP-bound, promotes receptor endocytosis (22, 23). It is tempting to speculate that increased expression of a Rab5 GAP such as PRC17 would lead to the formation of a relatively more inactive, GDP-bound form of Rab5, inhibit receptor endocytosis, and thereby prolong growth factor signaling and promote tumorigenesis. Enthusiasm for this proposal is tempered by the finding that a tumor suppressor gene, *TSC2*, also encodes a Rab5 GAP (24, 25). In this case, loss of Rab5 GAP activity is linked to tumorigenesis, in direct opposition to the effects we have observed with PRC17. Clearly, there is more to be learned about how PRC17 and TSC2 affect Rab5 function, endocytosis, and tumorigenic phenotypes.

We have provided several lines of evidence to demonstrate onco-

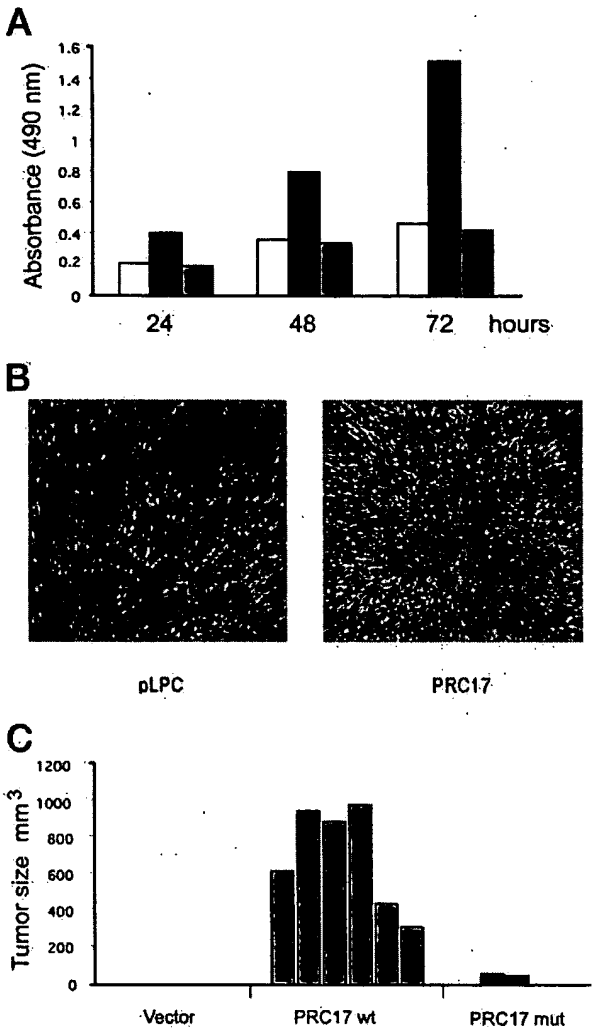

Fig. 3. Oncogenic properties of PRC17. *A*, cell proliferation assay. Cell growth rate is expressed as absorbance at 490 nm. 3T3-pLPC, □; 3T3-PRC17 wild type, ■; 3T3 PRC17 mutant, ▤. Data are representative of two independent experiments (n = 8 for each experiment). *B*, focus formation assay. 3T3 cells were transfected, split, and maintained in reduced serum medium. Foci started to appear after 7 days, when the cells reached confluence. Plates were monitored every day to determine when to score the foci formation. The photographs shown were taken at day 17. *C*, tumor tumorigenicity assay. 3T3 cells stably transfected with vector alone or wild-type or mutant PRC17 (5×10^6 cells) were injected into nude mice (n = 5). Tumor formation was monitored. The tumors were measured each week at their longest points using a caliper.

genic properties of PRC17. We showed that PRC17 confers a growth advantage to NIH3T3 cells in low serum, reduces contact inhibition, increases cellular invasion, and induces tumorigenesis in nude mice. Loss of PRC17 GAP activity resulted in loss of its transforming activity. Future studies will provide insight into the effects of PRC17 on Rab5 function and prostate cancer formation.

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